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ANTIMICROBIAL PEPTIDES WITH DIFFERENTIAL BACTERIAL BINDING CHARACTERISTICS

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14. ABSTRACT

This report documents research conducted by Natick Soldier Research, Development and Engineering Center (NSRDEC), between 2002 and 2010, to discover, design, and assess the differential bacterial binding ability of short antimicrobial peptides (AMPs) as part of an effort to provide rapid, accurate, and highly sensitive detection of bacterial contamination in Soldier wounds, food, and water sources. This detection capability is pivotal to maximize Warfighter survivability and quality of life. Current biosensor platforms incorporate recognition elements, such as antibodies, that are highly selective but have limited stability and sensitivity. The current drawbacks of the biosensor have limited its usefulness for rapid, real-time detection in an operational environment. Toward development of more robust and sensitive recognition elements, a series of truncated AMPs (7-15 amino acid residues) was designed using three methods: 1) fragmentation of naturally-occurring AMP sequences, 2) scanning alanine mutagenesis (applied to three fragments), wherein each residue in the sequence is systematically replaced with alanine to produce a set of well-defined mutations, and 3) sequence generation via a Markov chain algorithm utilizing a database of naturally-occurring AMPs. The fragmentation approach yielded multiple sequences possessing binding to all three organisms; additionally, several fragments exhibited selectivity for *E. coli* O157:H7 relative to *S. aureus*. Several of the fragments screened also displayed discriminatory binding to pathogenic *E. coli* O157:H7 relative to non-pathogenic *E. coli* ML35. The three fragments that were further engineered via scanning alanine mutagenesis had elevated binding responses and differential binding characteristics. Sequence generation via Markov chain also yielded peptides capable of selectivity between *E. coli* and *S. aureus*.

15. SUBJECT TERMS

BINDING	BIOSENSORS	ESCHERICHIA COLI	BACTERIAL PATHOGENS
PEPTIDES	ANTIBODIES	WOUNDS AND INJURIES	STAPHYLOCOCCUS AUREUS
BACTERIA	SURVIVABILITY	BIOLOGICAL DETECTION	
REAL TIME	QUALITY OF LIFE	ELISA(ENZYME LINKED IMMUNOSORBENT ASSAY)	
DETECTION	CONTAMINATION		

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Preface

This report documents the methods used in and the results from two research programs conducted at the Natick Soldier Research, Development and Engineering Center (NSRDEC) to identify and investigate the differential binding behavior of short antimicrobial peptide sequences. The first program, "Peptide Arrays for the Detection of Pathogenic Bacteria," was conducted between October 2002 and September 2005 and was funded under program element number AH52. The second program, "Peptide-Based Arrays for Detection of Bacterial Pathogens and Viruses," was conducted between January 2008 and December 2010 and was funded by a grant (number 8.10012_08_NRL_B) from the Defense Threat Reduction Agency (DTRA) Joint Science & Technology Office for Chemical and Biological Defense (JSTO-CBD).

Results from the above programs have been communicated through a proceeding in *Proceedings of the 24th Army Science Conference* [1], a book chapter in Microbial Surfaces: Structure, Interactions, and Reactivity [2], and two peer-review manuscripts, one in *Protein & Peptide Letters* [3], and the other in *Colloids and Surfaces B: Biointerfaces* [4]. While those publications focused on utilizing antimicrobial peptides found in nature, this report covers subsequent research focusing on shorter, engineered peptides. The goal of this research effort is to discover peptide sequences with differential binding behavior toward select microorganisms.

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Executive Summary

Between 2002 and 2010, the Natick Soldier Research, Development & Engineering Center (NSRDEC) conducted several studies to assess the differential bacterial binding ability of short antimicrobial peptides (AMPs) as part of an effort to provide rapid, accurate, and highly sensitive detection of bacterial contamination in Soldier wounds, food, and water sources. This detection capability is pivotal to maximize Warfighter survivability and quality of life. Current biosensor platforms incorporate recognition elements, such as antibodies, that are highly selective but have limited stability and sensitivity.

AMPs are biological molecules found in nature as part of the innate immune defense system in many organisms[1]. Hundreds of AMPs have been discovered to date, each with the ability to combat and prevent bacterial infections. Although most AMPs are active against a broad range of bacteria, the specificity and mechanism of their antimicrobial action varies between AMPs[2]. Most AMPs need to reach a threshold concentration in order to disrupt the cell membrane, leading to cell death. However, peptides will bind to the surface of the cell at lower concentrations, in some cases even binding to bacteria for which there is little or no antimicrobial capability[3]. The inherent peptide binding affinity for cell membranes was the basis of the NSRDEC research. The goal of these efforts was to develop a set of peptide sequences that can discriminate between various bacterial organisms through differential binding responses.

NSRDEC designed a series of small peptides, based on naturally-occurring AMPs, using three approaches. First, naturally-occurring AMP sequences were divided into fragments of 7-15 amino acid residues in length. Next, for three of the fragment sequences, PGQ_2_c, PL_1_c, and SMAP_2_c, individual amino acids were replaced with alanine (in a process called scanning alanine mutagenesis) to explore the effects of tailored sequence modifications on the binding properties of the peptide. Lastly, a Markov chain algorithm was developed to predict the probability of two amino acid residues appearing next to one another using naturally-occurring AMPs as a basis. The algorithm was used to generate unique peptide sequences with physical properties similar to those of naturally-occurring AMPs.

The peptides were screened for binding to pathogenic and non-pathogenic *Escherichia coli* (a Gram-negative bacterium) as well as *Staphylococcus aureus* (a Gram-positive bacterium). Normalized binding results were compared in order to discover peptides with binding behavior that was Gram-specific (*E. coli* vs. *S. aureus*) or strain-specific (pathogenic vs. non-pathogenic *E. coli*). Several of the peptide fragments demonstrated the ability to discriminate between pathogenic and non-pathogenic *E. coli*, signifying strain-specific binding behavior. Select fragments bound to *S. aureus*; however, an elevated binding response to *E. coli* was still evident, indicating Gram-specific binding for *E. coli*. The use of scanning alanine mutagenesis showed that both the overall binding response and binding specificity of the peptides is altered due to a single amino acid substitution. This process was used to enhance fragment PGQ_2_c's preference for pathogenic *E. coli* over the non-pathogenic strain, as well as to enhance fragment PL_1_c's discriminatory binding for *E. coli* relative to *S. aureus*. Several peptides designed using the Markov chain

[1] Tossi, A., Sandri, L., & Giangaspero, A. (2000). Amphipathic, α-Helical Antimicrobial Peptides. *Biopolymers, 55*, 4-30.

[2] Epand, R. M., & Vogel, H. J. (1999). Diversity of antimicrobial peptides and their mechanisms of action. *Biochimica et Biophysica Acta, 1462*, 11-28.

[3] Steiner, H., Andreu, D., & Merrifield, R. B. (1988). Binding and action of cecropin and cecropin analogues: antibacterial peptides from insects. *Biochimica et Biophysica Acta, 939*, 260-266.

algorithm exhibited binding to *E. coli* only, while one such peptide demonstrated binding only to *S. aureus*. Further modifications of the Markov chain algorithm to generate sequences with more specific physical properties yielded three additional peptides with binding to *S. aureus*.

The results of these studies lay the foundation for future efforts focusing on expanded screening of peptide fragment binding against additional Gram-negative and Gram-positive bacteria to more fully define the range of the fragment binding affinities. Due to the unique binding spectrum of each AMP fragment, an array of such peptides may be able to identify a given bacterium via a unique patterned binding signature. Ultimately, these peptides could be used as a new generation of molecular recognition elements to capture and detect pathogenic bacteria within a biosensor platform.

ANTIMICROBIAL PEPTIDES WITH DIFFERENTIAL BACTERIAL BINDING CHARACTERISTICS

1. Introduction

This report details efforts by the Natick Soldier Research, Development and Engineering Center (NSRDEC) to assess the feasibility of using short antimicrobial peptide (AMP) sequences as recognition elements to address the limitations of current biosensor technology in identifying select bacteria. The work was performed by NSRDEC under two research programs on AMP binding behavior. The goal of these programs was to discover peptide sequences with differential binding behavior toward select microorganisms. The first program, conducted between 2002 and 2005 and funded internally by NSRDEC, examined the binding of naturally-occurring AMPs and their sequence fragments with a focus on differential affinity toward pathogenic strains of bacteria. The efforts regarding naturally-occurring AMPs have been published in four peer-review manuscripts: *Proceedings of the 24th Army Science Conference* [1], a book chapter in *Microbial Surfaces: Structure, Interactions, and Reactivity* [2], *Protein and Peptide Letters* [3], and *Colloids and Surfaces B: Biointerfaces* [4]. Only the subsequent research from the first program, which focused on shorter AMPs engineered from the native sequences, is reported here. The second program, conducted between 2008 and 2010, expanded the study of sequence fragments to include several more parent peptides and additional custom sequences with the goal of determining binding behavior toward multiple pathogenic organisms. It was funded by the Defense Threat Reduction Agency (DTRA) Joint Science & Technology Office for Chemical & Biological Defense (JSTO-CBD).

The Army's research emphasis on identification of biological warfare agents, detection of food and water contamination, and prevention of wound infection for the Warfighter highlights the importance of real-time detection of infectious agents in the field. However, current detection systems lack the stability to perform outside a laboratory setting or are too cumbersome to deliver results in a timely manner. Commonly-explored biosensor systems, such as polymerase chain reaction (PCR) or antibody systems, are highly specific to target pathogens; however, the constraints of both systems limit their usefulness in an operational environment. PCR-based biosensors require time for cycles of DNA replication, which delays identification of the target analyte. DNA replication is also prone to contamination, necessitating support from a laboratory to produce accurate results. Antibody-based biosensors possess limited capability to detect low concentrations of pathogens due to the relatively large size of the antibody molecules. These limitations highlight the need for an alternative approach to pathogen detection using a novel class of recognition elements.

AMPs are part of the innate immune defense system in all higher organisms [5]. AMPs exhibit broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria, viruses, and fungi [6]. Hundreds of AMPs have been discovered and investigated to date. They are divided into several classes according to structure and amino acid composition, one of the most abundant being AMPs that form an α-helix [5]. The majority of cationic α-helical antimicrobial peptides exhibit lytic action that occurs via a two-step process: 1) an initial association with the cell wall and 2) disruption of the cell membrane when the AMPs reach a critical concentration. The nature and spectrum of the initial association of α-helical AMPs to the cell wall, typically an electrostatic or hydrophobic binding interaction, highlights the

peptides' potential as the basis for the design of new molecular recognition elements that will overcome current state-of-the-art limitations. Unlike antibodies and PCR products, AMPs are stable under a wide spectrum of pH and temperature conditions, allowing them to remain effective in an operational environment. AMPs are also small molecules relative to antibodies, allowing for incorporation of a relatively high concentration of AMPs into a biosensor platform and potentially increasing biosensor sensitivity.

In contrast to the specificity of antibodies and PCR products, AMPs exhibit binding affinity to a broad spectrum of infectious agents, including some of those to which they exhibit minimal antimicrobial activity [7]. However, several researchers have shown that the full sequence of many naturally-occurring AMPs is not necessary for both binding and antimicrobial activity [1,5-10]. Modified AMPs with N-terminal or C-terminal deletions retain their binding characteristics and antimicrobial activity [5,7,8]; however, after a certain number of deletions, either binding response or antimicrobial activity is reduced [7,8]. This behavior suggests that AMPs exhibit dedicated "domains," or portions of the sequence, for binding [1,10] or antimicrobial activity [8,9]. The activity domains of several AMPs have been shown to be species-specific [8,9]; however, little research has been performed to date on the specificity of binding domains.

Previous research at NSRDEC has focused on studying naturally-occurring AMP discriminatory binding. Gregory *et al*. [11] demonstrated preferential binding for *E. coli* O157:H7 relative to *E. coli* K12 through immobilization of the AMP cecropin P1 to amine-reactive microplates. Cecropin P1 was randomly immobilized through amino acids with inherently-free amine groups. Conversely, Soares *et al*. [3] modified a series of AMP sequences with a C-terminal cysteine residue for site-directed immobilization to a microplate with a thiol-reactive coating. Several peptides exhibited preferential binding for *E. coli* O157:H7 relative to *E. coli* ML35, which was maintained or enhanced under various pH and ionic strength conditions. These studies demonstrated promise of the use of full-length peptides as molecular recognition elements, but binding selectively was not sufficient. In addition, current methods to synthesize naturally-occurring AMP sequences are prohibitively expensive for the purposes of high-throughput screening and large-scale production. Therefore, focus has shifted to peptide fragmentation as a means to overcome the limitations seen with full-length peptides.

Over the last decade, an increasing amount of research has focused on shorter AMP sequences [5,12-14]. NSRDEC's efforts herein involve AMP "fragments," or targeted domains of the native α-helical AMP sequences, which were investigated to define the cell binding domains. Additionally, a series of short sequences was designed based on residue combinations found in naturally-occurring AMPs. The candidate fragments and short peptides were immobilized and assessed for their Gram-specific and strain-specific binding behavior. Short AMPs have the potential to be used as recognition elements that will address the shortcomings of current biosensor technology; this report details the initial efforts at NSRDEC to assess their feasibility.

2. Materials and Methods

2.1 Design of Peptide Fragments

2.1.1 Fragments Derived from Native Sequences

A database of AMPs was developed from the literature to classify candidate peptides based on sequence, activity, and structure [3]. The sequences chosen for fragmentation are listed in Table 1. Criteria considered for down-selection were a lack of post-translational modifications, the absence of cysteine residues, and antimicrobial activity against Gram-positive and/or Gram-negative bacteria. Overlapping peptide fragments of 7-15 amino acid residues in length, designed to include amino acid residues known to be important for antimicrobial activity (and presumably for binding as well), were developed from the selected AMP sequences. A cysteine residue was added to the C-terminus of each fragment sequence to enable site-directed immobilization. See Appendix A for a complete list of derived fragments.

Table 1: Naturally-occurring and Hybrid AMP Sequences Utilized for Design of Fragments

Peptide	Sequence	Antimicrobial Activity	
		Gram +	Gram -
PGQ [15]	GVLSNVIGYLKKLGTGALNAVLKQ	Weak	Moderate
Pleurocidin [16]	GWGSFFKKAAHVGKHVGKAALTHYL	Moderate	Moderate
SMAP 29 [17]	RGLRRLGRKIAHGVKKYGPTVLRIIRIAG	Strong	Strong
Cecropin A, *A. albopictus* [18]	GGLKKLGKKLEGVGKRVFKASEKALPVAVGIKALG	n.d.	qual. [19]
Cecropin P1 [20]	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR	Weak	Strong
Chrysophsin 1 [21]	FFGWLIKGAIHAGKAIHGLIHRRRH	Strong	Moderate
Chrysophsin 3 [21]	FIGLLISAGKAIHDLIRRRH	Moderate	Moderate
CA-MA [22]	KWKLFKKIGIGKFLHLAKKF	Strong	Strong
HP-ME [23]	AKKVFKRLGIGAVLKVLTTG	Strong	Strong
Strong activity: MIC ≤ 10 μM; Moderate activity: MIC = 10-100 μM; Weak activity: MIC ≥ 100 μM; n.d. = no data available; qual. = qualitative assessment of activity only.			

2.1.2 Fragment Enhancement via Scanning Alanine Mutagenesis

Peptide fragments PGQ_2_c, PL_1_c, and SMAP_2_c were further engineered via scanning alanine mutagenesis, wherein single amino acids were systematically replaced with an alanine residue to generate a series of alanine variants of the parent peptide fragment.

2.1.3 AMP-Inspired Sequences

To create a library of peptide sequences, the full-length AMPs in Table 1 were analyzed using a first-order Markov chain algorithm. Briefly, this algorithm predicts the probability of a given item in a series

appearing next to the previous one; in this instance, the algorithm predicted the likelihood of one amino acid appearing next to another in an AMP sequence. Using this algorithm, a probability table for pairs of amino acids appearing adjacent to one another was developed. This probability table, which included the predictions of sequence start and end points, was used to generate new sequences based on the weight given to each amino acid pair. Peptide sequences generated using this algorithm displayed similar hydrophobicity, charge, and amino acid motif characteristics to the native sequence-based fragments, but their sequences were otherwise random. For ease of synthesis, no residue was allowed to repeat more than three times, and sequences outside the range of 7-15 residues were discarded.

2.2 Peptide Synthesis, Characterization, and Preparation

2.2.1 Peptide Synthesis

Peptide fragments were synthesized via 9-fluorenylmethoxycarbonyl (FMOC) Solid-Phase Peptide Synthesis [24] using a 396Ω automated peptide synthesizer (AAPPTec, Louisville, KY) and were cleaved from the resin using Reagent K (5% phenol, 5% water, 5% thioanisole, 2.5% ethanedithiol (EDT), 82.5% trifluoroacetic acid (TFA; Sigma-Aldrich)). The majority of the fragments were resolubilized in 0.05% TFA/0.05% acetonitrile/water or 0.1% TFA/water, but organic solvents were used to assist solubilization in some cases (see Appendix A, Table A1 for a complete list of solvent combinations). Successful synthesis of each peptide was confirmed by using ElectroSpray Ionization-Mass Spectrometry (ESI-MS; Thermo Fisher Scientific, Waltham, MA) to compare theoretical and actual molecular weight.

Full-length AMPs, additional peptide fragments, and Markov chain sequence peptides were obtained from New England Peptide (Gardner, MA). Peptide fragments and Markov chain sequence peptides were synthesized in 1-2 mg quantities on a 96-well microtiter plate platform. Synthesis was confirmed via Reverse Phase-High Pressure Liquid Chromatography (RP-HPLC; Waters Corporation, Milford, MA) and/or Matrix Assisted Laser Desorption Ionization-Mass Spectrometry (MALDI-MS). Peptides were stored long-term in powder form at -20 °C and were solubilized in 0.1% TFA/water for purification and short-term storage at 4°C.

2.2.2 Peptide Purification

Synthesized peptides were assessed for initial purity via RP-HPLC using a Delta Pak C18 5 µm, 100Å column (Waters Corporation) with 0.1% TFA/acetonitrile as a mobile phase. (Impurities likely consisted primarily of sequences with surplus and/or missing amino acids.) Percent purity was calculated as a ratio of the area of the dominant peak relative to the total area of all peaks present. Peptide fragments with 85% or greater initial purity were considered suitable for screening.

Select peptide fragments were purified via Solid-Phase Extraction (SPE) using a C18 reverse-phase absorbant 96-cartridge microtiter plate (Waters Corporation). C18 cartridges were preconditioned with 1 mL of 0.1% TFA/acetonitrile, followed by 3-4 mL of 0.1% TFA/water. Peptides were bound to the absorbant and washed with 2% H_3PO_4. Increasing concentrations of acetonitrile in water (with 0.1% TFA) were applied to the absorbant, and the elutions were collected. Elutions of 0%, 5%, 25%, and 50% acetonitrile were collected for all peptides. In addition, elutions of 9%, 13%, and 17% were collected for New England Peptide batches NEP1, NEP2, and NEP3. The elutions were lyophilized and resolubilized in 0.1% TFA/water or PE (phosphate-buffered saline (PBS) [137 mM NaCl, 4.3 mM Na_2HPO_4•7H2O, 2.7 mM KCl, 1.4 mM KH_2PO_4] with 1 mM ethylenediaminetetraacetic acid (EDTA), pH 6.5). RP-HPLC,

under the conditions described previously, was used to determine the elution containing the target peptide and evaluate purity. In instances where multiple elutions contained the peptide, the elutions were combined.

Peptides ordered in bulk (≥ 5 g) from New England Peptide were purified by the company via HPLC. Batches NEP4 and NEP5 of the 96-well microplate orders of peptides (see Section 2.2.1) were purified by New England Peptide via SPE.

2.2.3 Quantitation and Reduction

The fragments PL_1_c, PGQ_2_c, SMAP_2_c, and CP1_4_c (parents of the scanning alanine mutagenesis libraries) were quantitated by amino acid compositional analysis (Commonwealth Biotechnologies, Inc., Richmond, VA). Standard curves for these four peptides were generated via RP-HPLC using the stationary and mobile phases described in Section 2.2.2. The scanning alanine mutagenesis libraries were quantitated using these standard curves as a reference. The remaining peptide fragments and Markov chain sequence peptides were quantitated via bicinchoninic acid (BCA) microplate protein assay (Thermo Fisher Scientific), per the manufacturer's instructions, with a fragment of known concentration, PGQ_2_c_7, as a standard.

Peptides were solubilized or diluted either in PED (PBS with 1 mM EDTA and 0.1 μM dithiothreitol), pH 6.5, or in PE, pH 6.5. Peptides in PED were considered reduced due to the presence of dithiothreitol and were stored at -20 °C. Peptides in PE were incubated with tris (2-carboxyethyl)phosphine (TCEP) in 3 molar excess. The peptide solutions in PE were flushed with N_2 gas for 30 s to remove oxygen, capped and incubated for 2 h at room temperature, and then incubated at 4° C overnight to complete reduction of disulfide bonds. These solutions were stored at 4 °C for short periods (2-3 weeks) and at -20° C for long-term storage. Freeze-thaw cycles were minimized to prevent disulfide bonds from re-forming.

2.3 Bacterial Cell Culture Preparation

E. coli O157:H7 (ATCC 43888), *E. coli* ML35 (ATCC 43827), and *Staphylococcus aureus* (ATCC 27217) were obtained from American Type Cell Culture Collection (ATCC; Manassas, VA). Cell cultures were grown in nutrient broth (*S. aureus*), tryptic soy broth (*E. coli* O157:H7), or luria broth (*E. coli* ML35). Cultures were grown to mid-log phase, measured as OD_{600nm} = 1 (approximately 10^8 CFU/mL). Cells were collected by centrifugation at 13,000 g for 5 min. The cell pellets were washed two times in PBS (pH 7.2) and resuspended in a volume equivalent to the original culture.

2.4 Assay Development and Screening

2.4.1 Determining Experimental Peptide Concentrations

The peptide concentration required to achieve saturation with native AMPs was determined using the method of Soares *et al*. [10]. Based on saturation curves of native AMPs, a peptide concentration of 15-25 mM was used for unpurified fragment binding studies. For purified fragment and AMP-inspired peptide binding studies, new saturation curves were developed using representative fragments, and a concentration of 30 μM was determined to sufficiently saturate the microplate wells. This concentration was used for purified fragment and AMP-inspired sequence binding studies.

2.4.2 Manual Whole Cell Binding Assays

Sulfhydryl-bind stripwell microplates with a maleimide reactive surface (Corning Inc., Corning, NY) were loaded with 100 µL of diluted peptide and incubated for 1 h while agitating at 875 rpm, 25 °C. Unbound peptide was removed by aspiration, and the wells were washed three times with PBS (pH 7.2). Each well was incubated with 150 µL 0.2% non-fat dried milk in PBS (pH 7.2) for 30 min without agitation to block any remaining active sites. The blocking solution was decanted, and 100 µL of PBS-prepared whole cells (10^8 CFU/mL) was added to the appropriate wells and incubated for 1½ h at 750 rpm, 25 ^{o}C. The unbound cell suspension was aspirated and wells were washed five times with PBS (pH 7.2). Detection of captured target bacteria was achieved by using a horseradish peroxidase (HRP)-conjugated antibody with affinity for the target organism (ViroStat, Portland, ME for *S. aureus* and *E. coli* ML35; Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD for *E. coli* O157:H7), diluted 1:1000 with 10% fetal bovine serum (FBS) in PBS (pH 7.2), and incubated for 1 h at 850 rpm. The unbound antibody solution was aspirated, and the wells were washed six times with PBS (pH 7.2). A two-component 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate system (Kirkegaard & Perry Laboratories Inc.) was prepared, and 100 µL of the preparation was added to each well, incubated for 30 min with gentle agitation at room temperature, and then scanned on a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA) at 650 nm.

2.4.3 Automated Whole Cell Binding Assays

Binding studies for purified fragments and AMP-inspired peptides were conducted using a Freedom EVO robotic platform (Tecan US, Inc., Durham, NC) with a GENios microplate reader (Tecan US, Inc.). The automated assay was designed to be identical to the manual assay with the following exceptions: all microplate agitation steps were conducted at 500 rpm, and wash steps utilized the PowerWasher 384 (Tecan US, Inc.), which simultaneously dispensed and aspirated PBS into the sample wells.

2.5 Data Analysis

2.5.1 Normalization

To account for non-specific binding of cells to the microplate, absorbance from a control absent of peptide was subtracted from the raw absorbance value. The results of this calculation are referred to as net values. To compensate for differences in antibody affinity for each organism, antibody normalization formulas were developed as described in a previous publication [2]. Briefly, antibody affinity curves were developed for each organism using varied cell concentrations. The affinity curve for *E. coli* O157:H7 was chosen as a baseline, and formulas were derived to correct for the observed absorbance from antibodies bound to an equal concentration of *E. coli* ML35 or *S. aureus* cells. The normalization formulas are:

E. coli ML35:

$$\ln y_{ML35(norm)} = 0.607 \ln y_{ML35(net)} + 1.579$$

S. aureus:

$$\ln y_{S.aureus(norm)} = 0.657 \ln y_{S.aureus(net)} + 0.156$$

Where $y_{(norm)}$ is the normalized value, and $y_{(net)}$ is the net absorbance value.

2.5.2 Differential Binding Behavior

The differential or discriminatory binding behavior of *E. coli* ML35 and *S. aureus* relative to *E. coli* O157:H7 are denoted as $D_{E.coli}$ and $D_{S.aureus}$, respectively. For a given peptide, these values were calculated by dividing the net binding response to *E. coli* O157:H7 by the normalized binding response to *E. coli* ML35 or *S. aureus*. Values greater than 1 indicate differential binding for *E. coli* O157:H7; values less than 1 indicate differential binding for *E. coli* ML35 or *S. aureus*, respectively.

3. Results and Discussion

3.1 Binding Behavior of AMP Fragments Derived from Native Sequences

Fragments were designed to elucidate the binding domains of the native AMPs for Gram-negative and Gram-positive organisms. The fragments were 9-14 amino acid residues long, and the sequences were designed to overlap at specific amino acids or amino acid motifs that were believed to be important for binding and/or antimicrobial activity [5]. Figure 1 shows an example of a naturally-occurring AMP, PGQ, and the fragments derived from that native sequence. Fragmentation of the remaining AMPs is detailed in Appendix A. Fragments of the AMPs cecropin P1 (CP1), PGQ, pleurocidin (PL), and SMAP-29 (SMAP), as well as their parent full-length peptides, were screened for binding to pathogenic and non-pathogenic *E. coli* as well as *S. aureus*. The screening studies facilitated understanding of bacterial binding domains within a full-length AMP for respective cells and enabled the discovery of fragments with preferential binding characteristics. Some fragments were not screened due to either unsuccessful synthesis or lack of solubility under the conditions described in Section 2.2.1.

```
                1        10        20
PGQ_c           GVLSNVIGYLKKLGTGALNAVLKQC
PGQ_1_c         GVLSNVIGYLC
PGQ_2_c                   KKGLTGALNAVC
PGQ_3_c               VIGYLKKLGTC
PGQ_4_c                         TGALNAVLKQC
```

Figure 1: Fragmentation of PGQ.
Fragments were designed to include residues known to be important for antimicrobial activity. C-terminal cysteine facilitates controlled immobilization.

In general, the peptide fragments displayed reduced binding to the organisms compared to their respective full-length parent AMPs (Figure 2; see Appendix B for numerical values and discriminatory binding data). Each set of fragments produced at least one sequence with binding to *E. coli* O157:H7, but only one fragment, PL_1_c, exhibited enhanced binding to the pathogen compared to the parent AMP. This fragment, derived from the N-terminus of PL, also exhibited comparable binding to *E. coli* ML35 and enhanced binding to *S. aureus* compared to the parent; however, it had clear discriminatory binding for *E. coli* O157:H7 relative to the other two organisms, with $D_{E.coli}$ = 1.677 (±0.2) and $D_{S.aureus}$ = 9.945 (±2.1). Indeed, the differential binding of PL_1_c for *E. coli* O157:H7 relative to *E. coli* ML35 was modestly greater than that of the parent ($D_{E.coli}$ = 1.272).

Compared to their parent sequences, several fragments exhibited a reduction or complete loss of discriminatory binding between the two strains of *E. coli*. The fragment PGQ_2_c exhibited the highest binding response for *E. coli* ML35 out of all fragments screened, and (accounting for normalization) it bound to *E. coli* O157:H7 with an equal level of response. While it had no significant differential binding between the two *E. coli* strains, PGQ_2_c had significant discriminatory binding for *E. coli* compared to *S. aureus* ($D_{S.aureus}$ = 3.586 ±0.9). It is worth noting that PGQ_2_c still exhibited the highest binding response for *S. aureus* out of all fragments screened. In addition to PGQ_2_c, fragments CP1_1_c, CP1_4_c, and PGQ_3_c exhibited a similar lack of differential binding between the two *E. coli* strains, albeit with much lower levels of binding overall.

Notably, several fragments exhibited discriminatory binding favoring *E. coli* ML35 relative to *E. coli* O157:H7. SMAP_5_c, from the middle of the native SMAP sequence, had a discriminatory binding of $D_{E.coli}$ = 0.377 (±0.1), while PL_4_c and SMAP_4_c had little to no binding to *E. coli* O157:H7. This

specificity is possibly due to the differences in lipopolysaccharide (LPS) between the two strains, specifically the O-antigen, which is present in *E. coli* O157:H7, but not the ML35 strain, and can affect the charge and hydrophobicity of the cellular membrane [3].

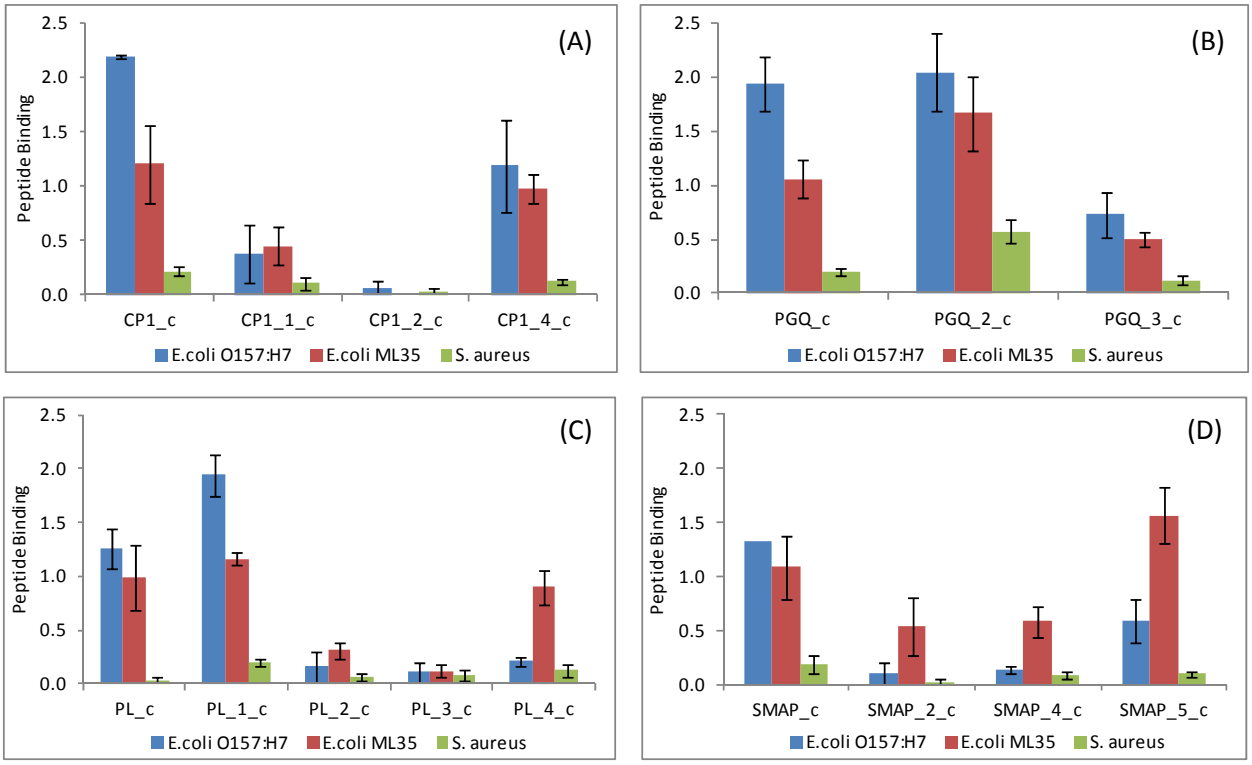

Figure 2: Cell-Binding Profiles for Native AMPs and Their Corresponding Fragments. (A) Cecropin P1, (B) PGQ, (C) Pleurocidin, (D) SMAP. Fragments PL_1_c and SMAP_5_c demonstrated the highest discriminatory binding between *E. coli* strains, while PGQ_2_c exhibited the highest overall binding response to all organisms screened. No fragments demonstrated discriminatory binding for *S. aureus*. Fragments were not purified prior to screening.

Additional peptide fragments were designed based on the AMPs chrysophsin 1 and 3 (CHRY1 and CHRY3), a *Helicobacter pylori* ribosomal protein L1 (2-9)-melittin (1-12) hybrid (HPME), and a *Hyalophora cecropia* cecropin A (1-8)-magainin 2 (1-12) hybrid (CAMA). Fragments based on these sequences, as well as CP1, PGQ, PL, SMAP, and *Aedes albopictus* cecropin A (CA), were synthesized by New England Peptide (batches NEP1, NEP2, and NEP3) and purified by SPE prior to screening. Due to low yields during both synthesis and purification of these initial batches, only a limited number of these fragments were characterized by screening assays. Figure 3 shows the binding responses of these fragments to *E. coli* O157:H7 and *S. aureus* (see Appendix B for numerical values and discriminatory binding data).

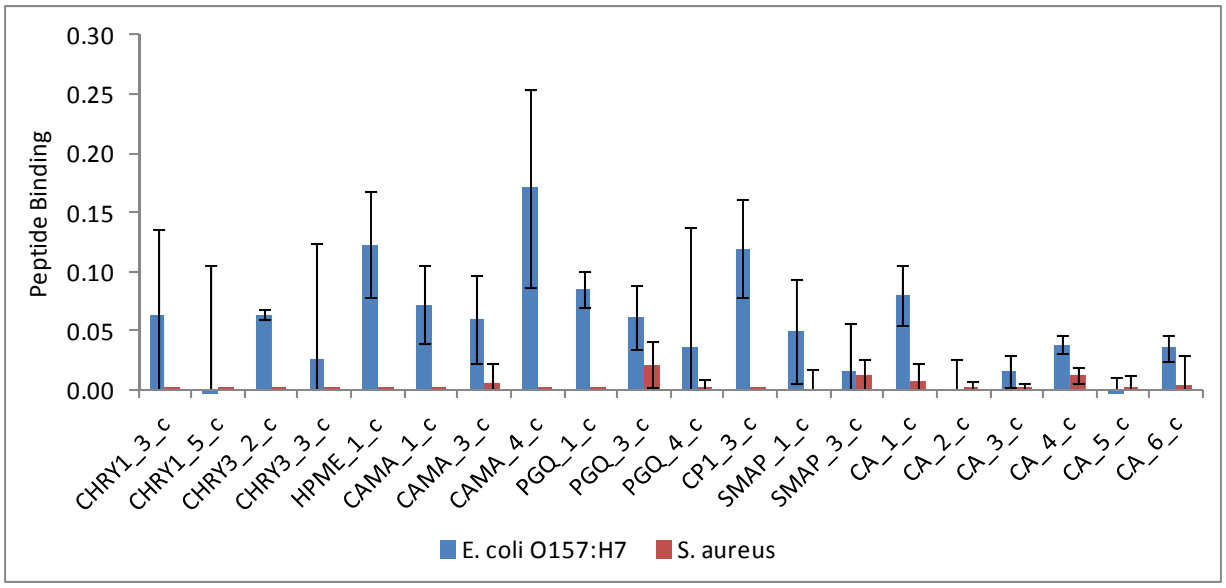

Figure 3: Binding Profiles of Peptide Fragments Synthesized by New England Peptide.

Despite the high variability within the binding data, several fragments (CHRY3_2_c, HPME_1_c, CAMA_1_c, CAMA_4_c, PGQ_1_c, and CP1_3_c) exhibited discriminatory binding for *E. coli* O157:H7 over *S. aureus*. Fragments were purified prior to screening.

High well-to-well variability was also observed in many samples. (The error bars in Figure 3 represent one standard deviation from the mean.) However, several fragments exhibited binding to *E. coli* despite the high variability. CAMA_4_c, which was derived from the C-terminal section of the AMP (i.e., the charged portion of melittin), exhibited the highest binding from this set of fragments. HPME_1_c, another fragment with discriminatory binding to *E. coli*, has a similar composition to CAMA_4_c: its sequence is highly positively charged (+4) and hydrophobic.

Despite the broad spectrum of antimicrobial activity demonstrated by the native peptides against both Gram-positive and Gram-negative bacteria in literature, none of the purified peptide fragments displayed significant binding to *S. aureus*. Since only a limited number of the fragments could be used in screening assays, it is possible that the Gram-positive binding domains of the native peptides were not represented among the fragments that were evaluated. It is also possible that an immobilized state negatively impacts the fragments' capacity to bind to *S. aureus*. Yet another possibility is that binding does not correlate directly to activity for Gram-positive bacteria, as seen in Figure 2, in which PL_c and SMAP_c each display poor overall binding to *S. aureus* despite possessing moderate to high activity against Gram-positive bacteria (Table 1). This contradiction may relate to peptide mechanism of inactivation for Gram-positive bacteria, which presumably differs from that described for Gram-negative bacteria.

3.2 Binding Behavior of Fragments Enhanced via Scanning Alanine Mutagenesis

Scanning alanine mutagenesis, the sequential replacement of amino acid residues in an AMP sequence with alanine, is a tool used to elucidate the importance of each individual amino acid and its effect on antimicrobial activity [5]. Here, the technique was used to understand the influence of individual amino acids and amino acid motifs on fragment binding. Alanine is a neutral, non-polar residue with a short side

chain, and its insertion into a sequence therefore alters the charge, hydrophobicity, and possibly the structural characteristics of the peptide. This method was applied to fragments PGQ_2_c, PL_1_c, and SMAP_2_c. Appendix C contains a complete list of the variants as well as their overall and discriminatory binding affinities (numerical values).

As seen in Section 3.1, PGQ_2_c did not exhibit discriminatory binding between *E. coli* O157:H7 and ML35; therefore, scanning alanine mutagenesis was applied to this fragment to further explore overall and differential binding between the two strains. The influence of scanning alanine mutagenesis on the peptide fragment PGQ_2_c binding behavior for the two *E. coli* strains is shown in Figure 4. Upon alanine substitution, several PGQ_2_c variants were created that exhibited enhanced discriminatory binding capability for *E. coli* O157:H7 (Table 2). This discrimination enhancement was primarily due to reduced binding to *E. coli* ML35.

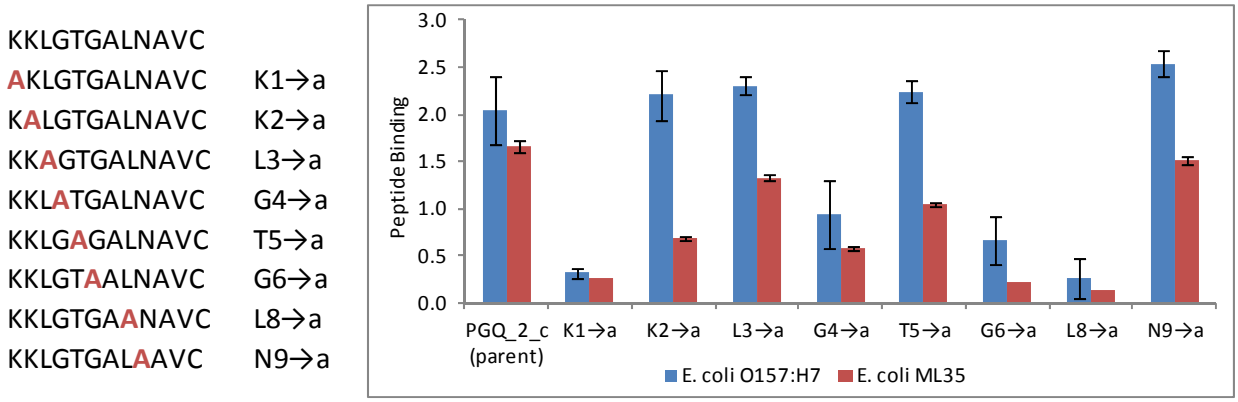

Figure 4: Sequence Modification and Binding Profiles for Scanning Alanine Mutagenesis Variants of PGQ_2_c.
Several amino acid substitutions resulted in binding decreases to one or both strains of *E. coli*. Variants were not purified prior to screening.

Alanine replacements of leucine in position 3, threonine in position 5, and asparagines in position 9 (denoted as L3→a, T5→a, and N9→a, respectively) exhibited similar binding profiles, demonstrating enhanced discriminatory binding for *E. coli* O157:H7 (42%, 75% and 42% increases, respectively). One of the variants, K2→a, was even more promising: it exhibited significantly enhanced discriminatory binding capability while maintaining high binding to *E. coli* O157:H7. The enhanced strain-specific binding observed in the K2→a variant (an increase of 167% compared to the parent) was primarily due to reduced binding to *E. coli* ML35. The K1→a variant exhibited similar reduced binding to *E. coli* ML3; however, binding to *E. coli* O157:H7 was similarly decreased, resulting in an overall limited binding response.

Table 2: Discriminatory Binding Ratios of PGQ_2_c Variants.

Variant	$D_{E.\ coli}$
Parent	1.2
K1→a	1.2
K2→a	3.2
L3→a	1.7
G4→a	1.7
T5→a	2.1
G6→a	3.1
L8→a	2.1
N9→a	1.7

While scanning alanine mutagenesis of PGQ_2_c did not produce variants with enhanced binding to either strain of *E. coli*, several of the variants exhibited severely reduced binding to both strains. Similar to K1→a, replacing the leucine in position 8 with alanine caused a near-total decrease in binding to both

strains. Binding was significantly reduced in the G4→a and G6→a variants, although G6→a exhibited enhanced discriminatory binding for *E. coli* O157:H7.

Alanine variants of PL_1_c were synthesized and screened against *E. coli* O157:H7 and *S. aureus*. Rather than to enhance discriminatory binding between two strains of *E. coli*, scanning alanine mutagenesis was applied to this fragment to manipulate discriminatory binding between *E. coli* O157:H7 and *S. aureus*. Unlike the variants of PGQ_2_c, this set of scanning alanine mutagenesis variants produced a sequence, S4→a, that possibly exhibited overall enhanced binding of both *E. coli* O157:H7 and *S. aureus* compared to the parent fragment (Figure 5). Several other variants, including G3→a and V12→a, may have displayed slightly enhanced binding to *E. coli* O157:H7 as well, but due to the large error bars in the *E. coli* binding data for this library, results were inconclusive.

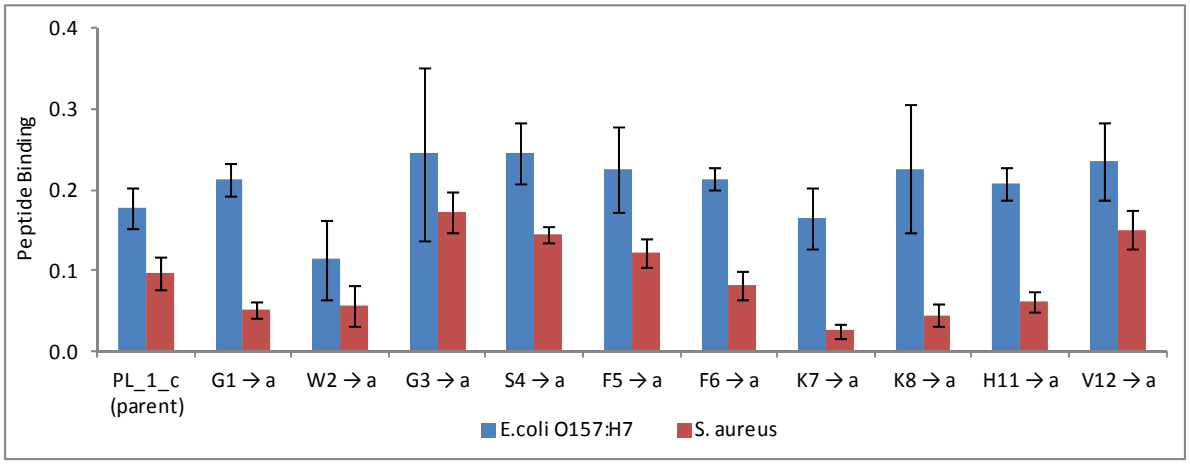

Figure 5: Binding Profiles for Purified Scanning Alanine Mutagenesis Variants of PL_1_c.

Several amino acid substitutions resulted in changes in binding response to *S. aureus*. Variants were purified prior to screening.

In contrast to the slight changes in binding to *E. coli*, the effects of amino acid substitution on binding to *S. aureus* were much more pronounced throughout the entire library. In addition to S4→a, two additional variants, G3→a and V12→a, exhibited a significant increase in binding, nearly double that of the parent in the case of G3→a. Although binding to *E. coli* was also enhanced in these fragments, each exhibited a partial loss of differential binding, particularly in the case of G3→a, which exhibited minimal differential binding capability. The variant F5→a maintained the discriminatory binding behavior of the parent; however, due to uncertainty in the *E. coli* data, the extent of its differential binding capability remains inconclusive.

Six variants exhibited reduced binding to *S. aureus* compared to the parent, including K7→a, which exhibited minimal binding to the organism. While two of these variants, W2→a and F6→a, only displayed modest increases in discriminatory binding (less than 50%), the remaining variants exhibited substantially enhanced

Table 3: Discriminatory Binding Ratios of PL_1_c Variants.

Variant	$D_{S.\ aureus}$
parent	1.8
G1 → a	4.1
W2 → a	2.0
G3 → a	1.4
S4 → a	1.7
F5 → a	1.8
F6 → a	2.6
K7 → a	6.2
K8 → a	5.0
H11 → a	3.4
V12 → a	1.6

discriminatory binding capability compared to parent PL_1_c (Table 3). The H11→a variant resulted in more than 80% enhancement, while the G1→a, K7→a, and K8→a variants resulted in well over 100% enhancement compared to the parent fragment. Because none of the variants displayed significantly increased binding to *E. coli* O157:H7 (with replacement of W2 with alanine actually causing binding to both organisms to decline), this enhanced discrimination was driven by the reduction in binding to *S. aureus*. The highest discriminatory binding was caused by replacement of K7 and K8, which are polar, charged residues (240% and 171% increases, respectively).

3.3 Binding Behavior of AMP-Inspired Sequences

A new set of short (7 to 15 residue) AMP-inspired sequences was designed using a Markov chain algorithm, which analyzed the native and hybrid AMP sequences in Table 1 to determine the probability of two amino acids appearing next to one another in a given sequence. An initial set of 96 short peptides produced using the Markov chain algorithm was purchased from New England Peptide (batch NEP4), and 48 were screened for binding to *S. aureus* and *E. coli* O157:H7. (The remaining sequences were too dilute or contained too many residual impurities for screening.) An additional 6 sequences from a subsequent batch (NEP5) were screened for binding to *S. aureus*. The majority of the sequences did not express binding to either organism; the full list of binding results can be found in Appendix D. Figure 6 contains a selection of sequences from this library that were of particular interest.

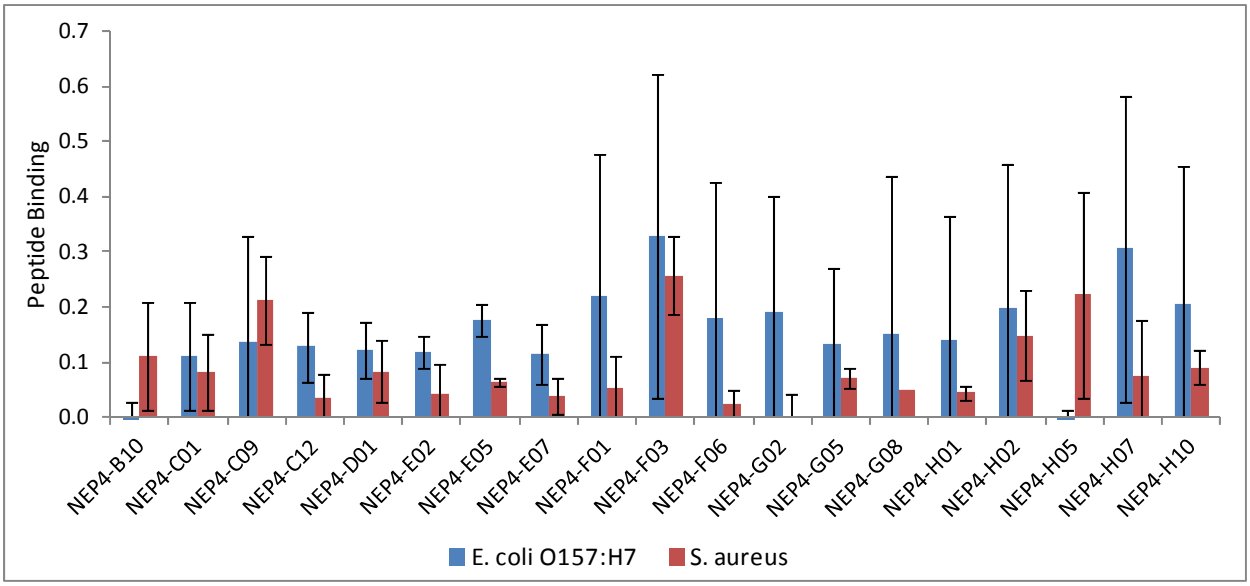

Figure 6: Binding Profiles of Selected Markov Chain Sequences.

Despite high variability in the binding data, several sequences exhibited binding to *E. coli*, and a few (NEP4-C09, NEP4-F03, and NEP4-H05) exhibited binding to *S. aureus*. Sequences omitted from this graph did not exhibit binding to either organism. Sequences were purified prior to screening.

As stated earlier, many of the peptides did not exhibit any binding to either *E. coli* or *S. aureus*, and several of those with binding only exhibited minimal response toward each organism. Analysis of these sequences was further complicated by the high variability of the binding data; as in the previous figures,

the error bars in Figure 6 represent one standard deviation from the mean (see Section 3.5 for further discussion on sample variability). However, over a dozen sequences displayed a low (≤ 0.1) but noticeable binding response to *S. aureus*, and several additional peptides were discovered that exhibited moderate to high (≥ 0.1) mean binding to *E. coli* O157:H7. Two of the *E. coli*-binding peptides, NEP4-C09 and NEP4-F03, also exhibited significant binding to *S. aureus*. These sequences displayed no significant differential binding (0.6 ±0.9 and 1.3 ±1.2, respectively). One sequence, NEP4-H05, exhibited binding only to *S. aureus*; however, high variability within the collected data on this peptide renders its level of differential binding difficult to determine.

The 54 AMP-inspired sequences possessed a broad range of peptide characteristics; however, NEP4-C09 and NEP4-F03, the two peptides with binding to both organisms, shared a high net charge (+5-6) and hydrophilicity, and both of their sequences contained at least three lysine residues. The *S. aureus*-binding NEP4-H05 (Figure 6) was less charged and more hydrophobic, but as it was the only sequence with possible discriminatory binding for *S. aureus*, it is difficult to determine whether these features are significant for binding behavior.

Additional sequences were designed using the Markov chain algorithm; however, these were filtered to possess one or more of the characteristics of peptides NEP4-C09 and NEP4-F03. A set of 17 sequences constrained to be partially hydrophilic and possess a high net charge (+4 or greater), and an additional 11 peptides with double- and triple-lysine motifs in their sequences, were synthesized by New England Peptide (batch NEP5) and screened for binding to *S. aureus*. As with the earlier AMP-inspired sequences, many of these constrained Markov chain sequences demonstrated minimal or a complete lack of binding to *S. aureus* (Figure 7). However, these new libraries produced a proportionately higher amount of sequences with *S. aureus*-binding capability than previous peptide synthesis batches. In addition, higher levels of binding to *S. aureus* were observed from the new sequences, particularly those with multiple-lysine motifs. Peptide NEP5-H01, from the multiple-

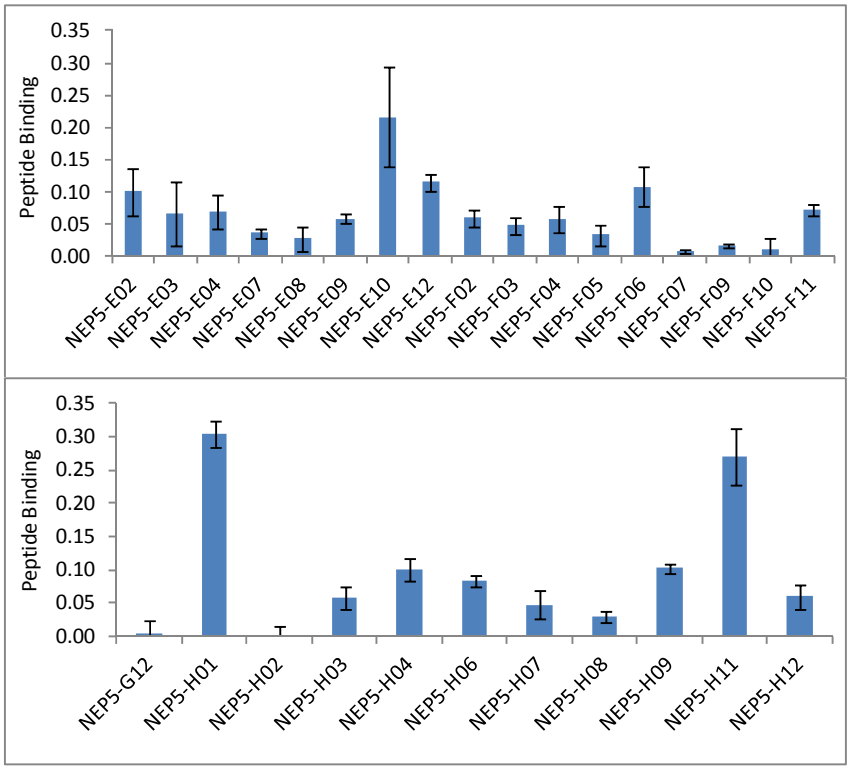

Figure 7: Binding Profiles of Constrained Markov Chain Sequences. The sequence library with constrained charge and hydrophobicity (top) produced one peptide (NEP5-E10) with significant binding to *S. aureus*. The library of sequences with double- and triple-lysine motifs (bottom) produced two peptides (NEP5-H01 and NEP5-H11) with good, statistically relevant binding for *S. aureus*.

lysine library, exhibited 19% greater binding to *S. aureus* than NEP4-F03. NEP5-E10, NEP5-H01, and NEP5-H11, the three sequences within these two new libraries that demonstrated the most significant binding to *S. aureus*, were all hydrophilic, exhibited a high net charge (+5 to +8), and were of similar length (13 to 14 amino acids in each sequence). These parameters alone are not sufficient to determine binding characteristics, since several sequences with less binding to *S. aureus* also exhibited these properties. The constrained Markov chain libraries have not been screened for their binding to *E. coli* O157:H7 due to low quantities of many of the peptides, so their differential binding behavior between the two organisms is still unknown.

3.4 Effect of Peptide Purification on Binding Behavior

Comparisons between the various crude (unpurified) and SPE-purified peptides in this report are difficult as most of the sequences were only screened in one state or the other (crude or purified). Direct analysis of the effect of purification, therefore, cannot be made for every peptide. The only library that was screened both before and after SPE-purification was the set of PL_1_c alanine variants. (See Appendix A for the parent PL_1_c sequence). The binding behavior of the purified sequences is discussed in Section 3.2. The values are repeated and compared with those from the crude sequences in Table 4 to illustrate the effects of purification on the binding behavior of each variant.

Table 4: Binding Behavior of Purified and Crude (Unpurified) PL_1_c Alanine Variants.

	***E. coli* binding**		***S. aureus* binding**		**$D_{S.\ aureus}$**	
	Crude	**Purified**	**Crude**	**Purified**	**Crude**	**Purified**
Parent	0.67 (±0.17)	0.18 (±0.03)	0.13 (±0.07)	0.10 (±0.02)	5.1 (±3.1)	1.8 (±0.5)
G1→a	0.47 (±0.13)	0.21 (±0.05)	0.15 (±0.06)	0.05 (±0.02)	3.2 (±1.5)	4.1 (±0.9)
W2→a	0.32 (±0.14)	0.11 (±0.02)	0.09 (±0.03)	0.06 (±0.01)	3.8 (±2.2)	2.0 (±1.3)
G3→a	0.89 (±0.07)	0.24 (±0.08)	0.12 (±0.06)	0.17 (±0.01)	7.3 (±3.8)	1.4 (±0.6)
S4→a	1.01 (±0.06)	0.25 (±0.04)	0.15 (±0.07)	0.14 (±0.01)	6.8 (±3.2)	1.7 (±0.3)
F5→a	0.36 (±0.10)	0.23 (±0.01)	0.11 (±0.04)	0.12 (±0.02)	3.3 (±1.4)	1.8 (±0.5)
F6→a	0.22 (±0.12)	0.21 (±0.05)	0.11 (±0.04)	0.08 (±0.02)	2.0 (±1.3)	2.6 (±0.6)
K7→a	0.19 (±0.12)	0.17 (±0.04)	0.08 (±0.03)	0.03 (±0.01)	2.4 (±1.8)	6.2 (±2.5)
K8→a	0.26 (±0.13)	0.23 (±0.11)	0.06 (±0.03)	0.05 (±0.03)	4.3 (±3.1)	5.0 (±2.3)
H11→a	0.41 (±0.14)	0.21 (±0.05)	0.06 (±0.02)	0.06 (±0.03)	6.8 (±3.6)	3.4 (±0.7)
V12→a	0.51 (±0.16)	0.24 (±0.02)	0.08 (±0.05)	0.15 (±0.01)	6.0 (±4.0)	1.6 (±0.4)

Most of the purified variants experienced reduced binding to *E. coli* O157:H7 compared to their crude counterparts. Surprisingly, the reduction was not uniform for all of the variants; S4→a experienced a 75% reduction, while K7→a and K8→a experienced less than 15% reduction. Binding to *S. aureus* also

decreased for six of the purified variants compared to the crude versions, but three of the variants, S4→a, F5→a, and H11→a, experienced no significant change, and two variants, G3→a and V12→a, actually demonstrated enhanced binding to *S. aureus* after purification.

The purpose of scanning alanine mutagenesis was to manipulate differential binding between *E. coli* O157:H7 and *S. aureus*, but purification appeared to change the effects of alanine substitution for each variant. Variants G3→a and S4→a exhibited the most enhanced binding to *E. coli*, and both had $D_{S.\ aureus} > 7$ in crude form. Once purified, and when compared to the parent peptide, they demonstrated enhanced binding to *S. aureus* with only a minimal difference in binding to *E. coli*. The resulting $D_{S.aureus}$ values of purified G3→a and S4→a were reduced to among the lowest of all of the PL_1_c variants evaluated. Differential binding capability of the parent fragment in purified form was also reduced (by 63%). Overall, several variants displayed enhanced discriminatory binding behavior when purified but not in crude form.

3.5 Assay to Assay Normalization and Error Analysis

Despite the use of controls, many of the peptides screened still exhibited considerable well-to-well and assay-to-assay variability. This was particularly an issue for the SMAP_2_c alanine variant library (data not shown) and many of the AMP-inspired peptides, and it hindered the collection of meaningful binding data. It was presumed that the peptides' positions on the microplate, as well as the limitations on the accurate range of the microplate reader, were responsible for the observed variability in binding. To investigate this presumption, two AMPs, CEME and PL, were screened against *E. coli* O157:H7 in multiple assays, using the same microplate layout each time. Next, another set of assays was conducted with the same AMPs, but with their positions on the microplate reversed. As seen in Figure 8, the binding affinities of the two AMPs were not affected by their position in the microplate. However, the PL sample sets exhibited relatively high standard deviations (40-50% of the mean), while CEME binding data exhibited variability less than 20% of the mean. Standard deviation comparisons within individual assays mirrored this trend (data not shown). The binding affinities of these AMPs were high enough that the plate reader could accurately detect color development; therefore, some other factor, as yet unknown, is responsible. These results suggest that assay error may be peptide-dependent rather than assay-related, but further analysis is warranted.

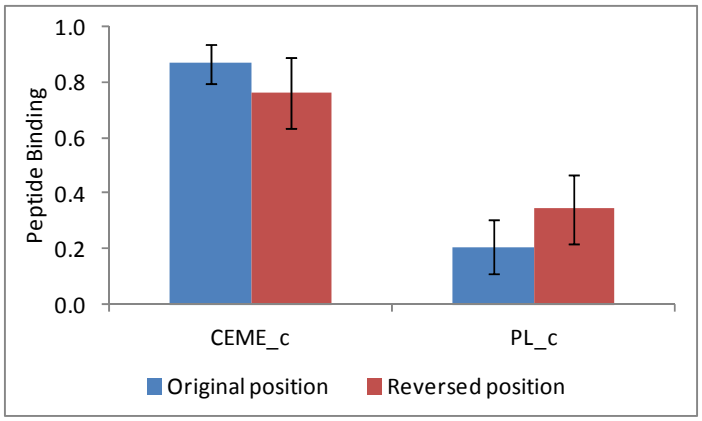

Figure 8: Comparison of Error in *E. coli* O157:H7 Screening Between the AMPs CEME and PL.

The standard deviation of the PL_c binding data is 40-50% of the mean, while the standard deviation of the CEME_c binding data is less than 20% ($N \geq 9$).

3.6 Considerations for AMP Design

While numerous reports have documented the general characteristics of peptides with antimicrobial activity, such as a positive net charge, an abundance of hydrophobic residues, and the propensity to form an amphipathic α-helix or β-sheet [1,2,5,25], less information is available on the relationship between

peptide characteristics and binding behavior. The results of scanning alanine mutagenesis variant screening in this report highlight the effect that replacing a single amino acid can have on peptide binding behavior. To effectively design AMPs with tailored binding properties in the future, the impact of these characteristics must be better understood.

One characteristic known to be important for peptide binding is positive net charge. Initial AMP binding takes place via electrostatic interactions between positively-charged amino acid residues and negatively-charged bacterial membrane lipids [25,26]. Replacing the lysine residue in position 1 of PGQ_2_c caused a near-total decrease in binding to both strains of *E. coli*, and replacing the lysine in position 2 also nearly eliminated binding to *E. coli* ML35 (Figure 4). It is unclear why replacement of the lysine in position 2 did not similarly affect binding to *E. coli* O157:H7; it's possible that only the lysine in position 1 is necessary for binding to that organism. The presence of two adjacent lysine residues appearing at or near the N-terminus was a recurring element observed in several additional fragments. Because the peptides are immobilized by the C-terminus, the amino acids near the N-terminus have the most potential to interact with cellular membranes. The presence of one or more lysines at the N-terminus likely facilitates interaction with the cells [26,27].

Hydrophobic interactions have also been shown to stabilize AMP binding [25-27], particularly to membranes with a weak negative charge [25]. The role of hydrophobicity helps to explain why fragments such as PGQ_1_c and CP1_3_c, two highly hydrophobic sequences, exhibited differential binding to *E. coli* O157:H7 despite their low net charges. Sequences such as SMAP_5_c, which has few hydrophobic residues but several charged residues, exhibited discriminatory binding to *E. coli* ML35 relative to *E. coli* O157:H7. In a recent publication, NSRDEC postulated that the AMP CP1 exhibits different mechanisms of binding in regard to the two *E. coli* strains; namely, CP1 binding to *E. coli* O157:H7 is due in part to hydrophobic interactions, while binding to *E. coli* ML35 is more primarily electrostatic [3]. This strain-dependent difference in peptide binding mechanism may explain why the fragment PL_1_c exhibited discriminatory binding to *E. coli* O157:H7 relative to *E. coli* ML35: the fragment's hydrophobic residues (comprising over 60% of its sequence) may facilitate binding to *E. coli* O157:H7 but not to *E. coli* ML35.

The AMPs listed in Table 1 all form an α-helix upon interaction with the Gram-negative bacterial cell membrane, a trait that is considered important for binding and activity in native AMPs [5-7,25]. Several fragments screened for binding affinity exhibited facial amphipathicity when projected onto helical wheels. Fragment CP1_4_c (Figure 9) has a large hydrophilic face, with several polar (white) and basic (yellow) residues, and only a small section populated by non-polar (blue) residues. Several other peptides with binding to one or more organisms also displayed distinct charged and hydrophobic faces on a helical wheel; however, others displayed little to no facial amphipathicity. The secondary structures of the peptide fragments have not yet

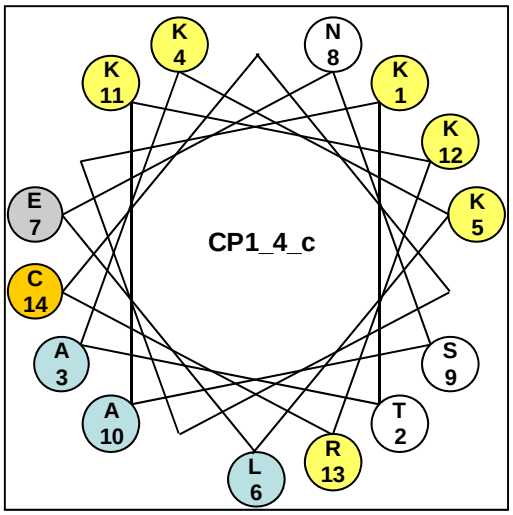

Figure 9: Helical Wheel Projection of CP1_4_c (Possible Structure).
Polar (white) and charged (yellow) residues are concentrated on one side of the helix, while the non-polar (blue) residues are clustered on the other side.

been analyzed, so their propensity to form an α-helix is not known. There is evidence, however, that for short AMPs, helicity is not necessary for antimicrobial activity [12,14]. It is possible that it is not necessary for binding, either; the apparent facial amphipathicity of these fragments may be coincidental. However, the tendency for the fragments with the highest overall binding responses to *E. coli* O157:H7, *E. coli* ML35, and *S. aureus* to contain a balance of polar and non-polar residues indicates that amphipathicity may be advantageous for binding.

While some changes in peptide binding behavior can be explained by the corresponding change in charge or hydrophobicity, other changes in binding behavior may be due to more nuanced differences between the side chains of each residue. The roles of individual amino acids and their interactions are still largely unknown and are added variables complicating efforts to design targeted cell-binding peptides in the future.

4. Conclusions

The capacity to tailor AMP sequences for discriminatory binding to specific targets highlights the potential to incorporate peptides as molecular recognition elements in a biosensor. Out of the sequences discussed in this report, the following were the most promising:

- **PL_1_c,** which demonstrated differential binding for *E. coli* O157:H7 relative to both *S. aureus* and *E. coli* ML35. The fragment exhibits differing binding responses to two pathogens, but it is also moderately capable of discriminating between a pathogenic and non-pathogenic strain of *E. coli*. Both categories of discrimination are advantageous characteristics in a molecular recognition element.
- **NEP4-H05,** which exhibited differential binding for *S. aureus* relative to *E. coli* O157:H7. Like PL_1_c, this AMP-inspired sequence demonstrated the capability to discriminate between a representative Gram-positive and Gram-negative pathogen, demonstrating its potential for use as a molecular recognition element.
- **PGQ_2_c,** which exhibited binding to all three organisms. Although peptides with tailored binding are required to identify pathogens, sequences with broad binding behavior would be useful as positive controls, i.e., as confirmation that the biosensor is functioning properly in instances when the target bacterial organisms are not present. The AMP-inspired sequence NEP4-F03 is potentially another candidate for this function.
- **SMAP_2_c, SMAP_4_c, and SMAP_5_c,** which displayed differential binding for *E. coli* ML35 relative to *E. coli* O157:H7. Like broad-binding peptides such as PGQ_2_c, sequences with discriminatory binding for a non-pathogenic strain relative to a pathogenic one would be useful controls in a biosensor. Peptides such as these would mitigate false-positive results due to the detection of non-pathogenic strains of a target bacterial species.

In order to achieve the stated goal of using AMPs as molecular recognition elements in a patterned array biosensor, much research must still be accomplished. Binding behavior to additional organisms, binding profiles for samples with organism cocktails, and the effects of a complex sample matrix on binding behavior will all need to be assessed in the future. The effects of cell concentration on peptide binding response and the structure of peptide fragments, both in solution and in an immobilized state, should also be studied. Additionally, despite efforts to normalize the sample data, some peptide sequences exhibited high well-to-well variability. This variability was likely a product of the sequences themselves, but it may be resolved when a more controlled surface, such as a self-assembled monolayer with thiol-reactivity, is developed. However, the results documented in this report are a significant first step in the development of short peptide sequences as recognition elements in a more versatile biosensor that can overcome limitations in the current state-of-the-art. Specific techniques and technical aspects of this work have transitioned to recent programs in the areas of aptamers for biosensing and bacteriocin discovery for tailored antimicrobials.

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List of Abbreviations and Acronyms

AMP:	antimicrobial peptide
ATTC:	American Type Culture Collection
BCA:	bicinchoninic acid
CA:	cecropin A
CAMA:	cecropin A (1-8)-magainin 2 (1-12) hybrid
CEME:	cecropin A-melittin hybrid
CHRY1:	chrysophsin 1
CHRY3:	chrysophsin 3
CP1	cecropin P1
E. coli:	*Escherichia coli*
EDT:	ethanedithiol
EDTA:	ethylenediaminetetraacetic acid
ELISA:	enzyme-linked immunosorbent assay
ESI-MS:	electrospray ionization-mass spectroscopy
FBS:	fetal bovine serum
FMOC:	9-fluorenylmethoxycarbonyl
HPLC:	see RP-HPLC
HPME:	*Helicobacter pylori* ribosomal protein L1 (2-20)-melittin (1-12) hybrid
HRP:	horseradish peroxidase
LPS:	lipopolysaccharide
MALDI-MS:	matrix assisted laser desorption ionization-mass spectroscopy
NSRDEC:	Natick Soldier Research, Development, and Engineering Center
PBS:	phosphate-buffered saline
PCR:	polymerase chain reaction
PE:	PBS with 1mM EDTA
PED:	PBS with 1mM EDTA and 0.1μM dithiothreitol
PEG:	polyethylene glycol
PL:	pleurocidin
RP-HPLC:	reverse phase-high pressure liquid chromatography
S. aureus:	*Staphylococcus aureus*
SMAP:	SMAP-29
SPE:	solid-phase extraction
TCEP:	tris (2-carboxyethyl)phosphine
TFA:	trifluoroacetic acid
TMB:	3,3',5,5'-tetramethylbenzidine
WCB:	whole cell binding assay

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Appendix A: Native AMP Fragments

(parent AMP names are in bold)

```
            1       10        20        30
CP1_c       SWLSKTAKKLENSAKKRISEGIAIAIQGGPRC
CP1_1_c     SWLSKTAKKLC
CP1_2_c               ENSAKKRISEGC
CP1_3_c                           IAIAIQGGPRC
CP1_4_c         KTAKKLENSAKKRC
CP1_5_c*                      ISEGIAIAIQC

            1       10        20
PGQ_c       GVLSNVIGYLKKLGTGALNAVLKQC
PGQ_1_c     GVLSNVIGYLC
PGQ_2_c               KKGLTGALNAVC
PGQ_3_c          VIGYLKKLGTC
PGQ_4_c                     TGALNAVLKQC

            1       10        20
CHRY1_c     FFGWLIKGAIHAGKAIHGLIHRRRHC
CHRY1_1_c*  FFGWLIKGAC
CHRY1_2_c*             AGKAIHC
CHRY1_3_c                  IHGLIHRRRHC
CHRY1_4_c*     LIKGAIHAC
CHRY1_5_c              HAGKAIHGLC

            1       10        20
CHRY3_c     FIGLLISAGKAIHDLIRRRHC
CHRY3_1_c*  FIGLLISAGKAC
CHRY3_2_c              IHDLIRRRHC
CHRY3_3_c        SAGKAIHDLIC

            1       10        20
PL_c        GWGSFFKKAAHVGKHVGKAALTHYLC
PL_1_c      GWGSFFKKAAHVC
PL_2_c                GKHVGKAALTC
PL_3_c           FKKAAHVGKHVC
PL_4_c                       GKAALTHYLC
```

```
            1       10        20        30
SMAP_c      RGLRRLGRKIAHGVKKYGPTVLRIIRIAGC
SMAP_1_c    RGLRRLGRC
SMAP_2_c            KIAHGVKKYGC
SMAP_3_c                        PTVLRIIRIAGC
SMAP_4_c          LGRKIAHGVC
SMAP_5_c                    KKYGPTVLRC

            1       10        20        30
CA_c        GGLKKLGKKLEGVGKRVFKASEKALPVAVGIKALGC
CA_1_c      GGLKKLGKKLC
CA_2_c                EGVGKRVFKASEKC
CA_3_c                                ALPVAVGIKALGC
CA_4_c           LGKKLEGVGKRC
CA_5_c                      VFKASEKALPC
CA_6_c                                  VAVGIKALGC

            1       10        20
CAMA_c      KWKLFKKIGIGKFLHLAKKFC
CAMA_1_c    KWKLFKKIC
CAMA_2_c*           GIGKFLHLC
CAMA_3_c               KFLHLAKKFC
CAMA_4_c          KKIGIGKFLC

            1       10        20
HPME_c      AKKVFKRLGIGAVLKVLTTGC
HPME_1_c    AKKVFKRLGIC
HPME_2_c*             GAVLKVLTTGC
HPME_3_c*       VFKRLGIGAC
HPME_4_c*           GIGAVLKVLC
```

*Fragments were not screened due to either difficulty with synthesis or lack of solubility.

Table A-1: Solubility and stock solution conditions for AMP fragments

Fragment	Lot #	Solvents (%)
CP1_1_c	JB08-16	TFA (0.05%), acetonitrile (0.05%), water (99.9%)
CP1_2_c	JB08-17	TFA (0.05%), acetonitrile (0.05%), water (99.9%)
CP1_4_c	JB04-5	TFA (0.05%), acetonitrile (0.05%), water (99.9%)
PGQ_2_c	JB04-12	TFA (0.05%), acetonitrile (0.05%), water (99.9%)
PGQ_2_c K1→a	MC01-29	TFA (0.1%), water (99.9%)
PGQ_2_c K2→a	MC01-28	TFA (0.1%), water (99.9%)
PGQ_2_c L3→a	MC01-27	TFA (0.1%), water (99.9%)
PGQ_2_c G4→a	MC01-25	TFA (0.1%), water (99.9%)
PGQ_2_c T5→a	MC01-23	TFA (0.1%), water (99.9%)
PGQ_2_c G6→a	MC01-19	TFA (0.1%), water (99.9%)
PGQ_2_c L8→a	MC01-18	TFA (0.1%), water (99.9%)
PGQ_2_c N9→a	MC01-17	TFA (0.1%), water (99.9%)
PGQ_3_c	JB04-13	TFA (0.05%), acetonitrile (0.05%), water (99.9%)
PL_1_c	JB05-12	TFA (0.05%), acetonitrile (0.05%), water (99.9%)
PL_1_c G1→a	MC02-27	TFA (0.1%), water (99.9%)
PL_1_c W2→a	MC02-28	TFA (0.1%), water (99.9%)
PL_1_c G3→a	MC02-29	TFA (0.1%), water (99.9%)
PL_1_c S4→a	MC02-32	TFA (0.1%), water (99.9%)
PL_1_c F5→a	MC02-33	TFA (0.1%), water (99.9%)
PL_1_c F6→a	MC02-34	TFA (0.1%), water (99.9%)
PL_1_c K7→a	MC02-35	TFA (0.1%), water (99.9%)
PL_1_c K8→a	MC02-36	TFA (0.1%), water (99.9%)
PL_1_c H11→a	MC02-37	TFA (0.1%), water (99.9%)
PL_1_c V12→a	MC02-38	TFA (0.1%), water (99.9%)
PL_2_c	JB04-15	2-propanol (33%), TFA (0.03%), acetonitrile (0.03%), water (67%)
PL_3_c	JB08-30	TFA (0.05%), acetonitrile (0.05%), water (99.9%)
PL_4_c	JB04-16	TFA (0.05%), acetonitrile (0.05%), water (99.9%)
SMAP_2_c	JB05-10	TFA (0.05%), acetonitrile (0.05%), water (99.9%)
SMAP_2_c K1→a	MC01-19	TFA (0.1%), water (99.9%)
SMAP_2_c I2→a	MC01-20	TFA (0.1%), water (99.9%)
SMAP_2_c H4→a	MC01-22	TFA (0.1%), water (99.9%)
SMAP_2_c G5→a	MC01-23	TFA (0.1%), water (99.9%)
SMAP_2_c V6→a	MC01-24	TFA (0.1%), water (99.9%)
SMAP_2_c K7→a	MC01-27	TFA (0.1%), water (99.9%)
SMAP_2_c K8→a	MC01-28	TFA (0.1%), water (99.9%)
SMAP_2_c Y9→a	MC01-29	TFA (0.1%), water (99.9%)
SMAP_2_c G10→a	MC01-30	TFA (0.1%), water (99.9%)
SMAP_4_c	JB04-10	TFA (0.05%), acetonitrile (0.05%), water (99.9%)
SMAP_5_c	JB05-11	TFA (0.05%), acetonitrile (0.05%), water (99.9%)

Note: All peptides synthesized at NEP were solubilized in 0.1% TFA / water.

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Appendix B: Screening Results of AMP Fragments

Table B-1: Binding behavior of AMP fragments

Lot #	Fragment	*E. coli* O157:H7	*E. coli* ML35	*S. aureus*
-	CP1_1_c[#]	0.382 (±0.260)	0.452 (±0.173)	0.109 (±0.055)
-	CP1_2_c[#]	0.068 (±0.070)	0.000 (±0.000)	0.034 (±0.027)
NEP2-B11	CP1_3_c	0.120 (±0.042)	-	0.000 (±0.000)
-	CP1_4_c[#]	1.191 (±0.424)	0.975 (±0.128)	0.123 (±0.026)
NEP3-B05	PGQ_1_c	0.085 (±0.016)	-	0.000 (±0.000)
-	PGQ_2_c[#]	2.046 (±0.360)	1.665 (±0.342)	0.571 (±0.110)
-	PGQ_3_c[#]	0.732 (±0.208)	0.498 (±0.071)	0.124 (±0.042)
NEP2-B07	PGQ_3_c	0.062 (±0.028)	-	0.022 (±0.020)
NEP1-B08	PGQ_4_c	0.036 (±0.101)	-	0.000 (±0.010)
-	PL_1_c[#]	1.942 (±0.198)	1.158 (±0.059)	0.195 (±0.036)
-	PL_1_c	0.178 (±0.026)	-	0.097 (±0.020)
-	PL_2_c[#]	0.162 (±0.144)	0.309 (±0.079)	0.065 (±0.039)
-	PL_3_c[#]	0.106 (±0.088)	0.117 (±0.060)	0.077 (±0.046)
-	PL_4_c[#]	0.211 (±0.045)	0.897 (±0.156)	0.128 (±0.060)
NEP3-C03	SMAP_1_c	0.050 (±0.044)	-	0.000 (±0.018)
-	SMAP_2_c[#]	0.111 (±0.093)	0.541 (±0.264)	0.033 (±0.024)
-	SMAP_2_c	0.078 (±0.052)	-	-
NEP3-C05	SMAP_3_c	0.015 (±0.041)	-	0.012 (±0.014)
-	SMAP_4_c[#]	0.147 (±0.032)	0.589 (±0.148)	0.092 (±0.039)
-	SMAP_5_c[#]	0.591 (±0.198)	1.567 (±0.260)	0.106 (±0.026)
NEP2-A03	CHRY1_3_c	0.063 (±0.028)	-	0.000 (±0.000)
NEP3-A05	CHRY1_5_c	0.000 (±0.106)	-	0.000 (±0.000)
NEP2-A07	CHRY3_2_c	0.064 (±0.004)	-	0.000 (±0.000)
NEP3-A08	CHRY3_3_c	0.025 (±0.099)	-	0.000 (±0.000)
NEP3-A09	HPME_1_c	0.123 (±0.044)	-	0.000 (±0.000)
NEP2-B01	CAMA_1_c	0.072 (±0.033)	-	0.000 (±0.000)
NEP1-B03	CAMA_3_c	0.060 (±0.037)	-	0.006 (±0.017)
NEP3-B04	CAMA_4_c	0.171 (±0.084)	-	0.000 (±0.000)
NEP3-C12	CA_1_c	0.080 (±0.026)	-	0.008 (±0.015)
NEP3-D01	CA_2_c	0.000 (±0.027)	-	0.000 (±0.007)
NEP3-D02	CA_3_c	0.016 (±0.013)	-	0.000 (±0.006)
NEP3-D03	CA_4_c	0.038 (±0.007)	-	0.012 (±0.007)
NEP3-D04	CA_5_c	0.000 (±0.010)	-	0.000 (±0.013)
NEP2-D05	CA_6_c	0.036 (±0.011)	-	0.004 (±0.025)

[#] Fragment not purified prior to screening.

Gray shading indicates confidence that peptide binds to target organism (confidence defined as mean binding value ≥ 0.050; mean binding value minus one standard deviation ≥ 0.020).

Dash indicates not evaluated.

Table B-2: Discriminatory binding capability of AMP fragments

Lot #	Fragment	$D_{E.\ coli}$*	$D_{S.\ aureus}$^
-	CP1_1_c#	0.8 (±0.7)	3.5 (±3.0)
-	CP1_2_c#	N/A	2.0 (±2.6)
NEP2-B11	CP1_3_c	-	N/A
-	CP1_4_c#	1.2 (±0.5)	9.7 (±4.0)
NEP3-B05	PGQ_1_c	-	N/A
-	PGQ_2_c#	1.2 (±0.3)	3.6 (±0.9)
-	PGQ_3_c#	1.5 (±0.5)	5.9 (±2.6)
NEP2-B07	PGQ_3_c	-	2.8 (±2.8)
NEP1-B08	PGQ_4_c	-	N/A
-	PL_1_c#	1.7 (±0.2)	9.9 (±2.1)
-	PL_1_c	-	1.8 (±0.5)
-	PL_2_c#	0.5 (±0.5)	2.5 (±2.7)
-	PL_3_c#	0.9 (±0.9)	1.4 (±1.4)
-	PL_4_c#	0.2 (±0.1)	1.7 (±0.8)
NEP3-C03	SMAP_1_c	-	N/A
-	SMAP_2_c#	0.2 (±0.2)	3.4 (±3.8)
-	SMAP_2_c	-	N/A
NEP3-C05	SMAP_3_c	-	1.2 (±3.6)
-	SMAP_4_c#	0.2 (±0.1)	1.6 (±0.8)
-	SMAP_5_c#	0.4 (±0.1)	5.6 (±2.3)
NEP2-A03	CHRY1_3_c	-	N/A
NEP3-A05	CHRY1_5_c	-	N/A
NEP2-A07	CHRY3_2_c	-	N/A
NEP3-A08	CHRY3_3_c	-	N/A
NEP3-A09	HPME_1_c	-	N/A
NEP2-B01	CAMA_1_c	-	N/A
NEP1-B03	CAMA_3_c	-	9.9 (±27.5)
NEP3-B04	CAMA_4_c	-	N/A
NEP3-C12	CA_1_c	-	10.1 (±19.4)
NEP3-D01	CA_2_c	-	N/A
NEP3-D02	CA_3_c	-	N/A
NEP3-D03	CA_4_c	-	3.1 (±1.9)
NEP3-D04	CA_5_c	-	N/A
NEP2-D05	CA_6_c	-	9.3 (±60.6)

* $D_{E.\ coli}$ = Discriminatory binding capability for *E. coli* O157:H7 relative to *E. coli* ML35.

^ $D_{S.\ aureus}$ = Discriminatory binding capability for *E. coli* O157:H7 relative to *S. aureus*.

N/A = No detectable binding to one or both organisms.

Fragment not purified prior to screening.

Gray shading indicates confidence in discriminatory binding (D_{cell} is more than one standard deviation away from 1.0; the peptide binds to at least one organism).

Dash indicates not evaluated.

Appendix C: Screening Results of Scanning Alanine Mutagenesis Variants

Table C-1: Binding behavior of alanine variants

Fragment	*E. coli* O157:H7	*E. coli* ML35	*S. aureus*
PGQ_2_c$^{\#}$	2.046 (±0.360)	1.665 (±0.342)	-
PGQ_2_c K1→a$^{\#}$	0.323 (±0.053)	0.265 (±0.135)	-
PGQ_2_c K2→a$^{\#}$	2.207 (±0.266)	0.690 (±0.235)	-
PGQ_2_c L3→a$^{\#}$	2.306 (±0.093)	1.333 (±0.179)	-
PGQ_2_c G4→a$^{\#}$	0.937 (±0.357)	0.580 (±0.240)	-
PGQ_2_c T5→a$^{\#}$	2.245 (±0.123)	1.047 (±0.214)	-
PGQ_2_c G6→a$^{\#}$	0.669 (±0.248)	0.219 (±0.111)	-
PGQ_2_c L8→a$^{\#}$	0.266 (±0.214)	0.130 (±0.108)	-
PGQ_2_c N9→a$^{\#}$	2.542 (±0.139)	1.509 (±0.260)	-
PL_1_c$^{\#}$	0.668 (±0.170)	-	0.130 (±0.070)
PL_1_c G1→a$^{\#}$	0.471 (±0.128)	-	0.149 (±0.058)
PL_1_c W2→a$^{\#}$	0.324 (±0.142)	-	0.085 (±0.033)
PL_1_c G3→a$^{\#}$	0.889 (±0.073)	-	0.122 (±0.062)
PL_1_c S4→a$^{\#}$	1.014 (±0.059)	-	0.149 (±0.069)
PL_1_c F5→a$^{\#}$	0.364 (±0.099)	-	0.112 (±0.036)
PL_1_c F6→a$^{\#}$	0.223 (±0.123)	-	0.111 (±0.035)
PL_1_c K7→a$^{\#}$	0.190 (±0.121)	-	0.079 (±0.031)
PL_1_c K8→a$^{\#}$	0.258 (±0.133)	-	0.060 (±0.029)
PL_1_c H11→a$^{\#}$	0.405 (±0.142)	-	0.059 (±0.024)
PL_1_c V12→a$^{\#}$	0.514 (±0.156)	-	0.085 (±0.051)
PL_1_c	0.178 (±0.026)	-	0.097 (±0.020)
PL_1_c G1→a	0.213 (±0.020)	-	0.052 (±0.010)
PL_1_c W2→a	0.114 (±0.050)	-	0.056 (±0.025)
PL_1_c G3→a	0.245 (±0.106)	-	0.173 (±0.025)
PL_1_c S4→a	0.246 (±0.038)	-	0.144 (±0.011)
PL_1_c F5→a	0.226 (±0.052)	-	0.122 (±0.018)
PL_1_c F6→a	0.214 (±0.014)	-	0.082 (±0.018)
PL_1_c K7→a	0.166 (±0.038)	-	0.027 (±0.009)
PL_1_c K8→a	0.227 (±0.080)	-	0.046 (±0.013)
PL_1_c H11→a	0.208 (±0.020)	-	0.062 (±0.012)
PL_1_c V12→a	0.235 (±0.048)	-	0.151 (±0.024)
SMAP_2_c	0.078 (±0.052)	-	0.000 (±0.041)
SMAP_2_c K1→a	0.118 (±0.062)	-	0.000 (±0.039)
SMAP_2_c I2→a	0.010 (±0.009)	-	-
SMAP_2_c H4→a	0.044 (±0.016)	-	0.000 (±0.037)
SMAP_2_c G5→a	0.053 (±0.022)	-	0.000 (±0.034)
SMAP_2_c V6→a	0.140 (±0.084)	-	0.000 (±0.051)
SMAP_2_c K7→a	0.172 (±0.085)	-	0.000 (±0.040)
SMAP_2_c K8→a	0.070 (±0.060)	-	0.000 (±0.041)

Fragment	*E. coli* O157:H7	*E. coli* ML35	*S. aureus*
SMAP_2_c Y9→a	0.070 (±0.039)	-	0.000 (±0.043)
SMAP_2_c G10→a	0.105 (±0.090)	-	0.000 (±0.069)

[#] Fragment not purified prior to screening.

Gray shading indicates confidence that peptide binds to target organism (confidence defined as mean binding value ≥ 0.050; mean binding value minus one standard deviation ≥ 0.020).

Dash indicates not evaluated.

Table C-2: Discriminatory binding capability of alanine variants

Fragment	$D_{E.\ coli}$*	$D_{S.\ aureus}$^
PGQ_2_c[#]	1.2 (±0.3)	-
PGQ_2_c K1→a[#]	1.2 (±0.7)	-
PGQ_2_c K2→a[#]	3.2 (±1.2)	-
PGQ_2_c L3→a[#]	1.7 (±0.2)	-
PGQ_2_c G4→a[#]	1.6 (±0.9)	-
PGQ_2_c T5→a[#]	2.1 (±0.5)	-
PGQ_2_c G6→a[#]	3.1 (±1.9)	-
PGQ_2_c L8→a[#]	2.1 (±2.4)	-
PGQ_2_c N9→a[#]	1.7 (±0.3)	-
PL_1_c[#]	-	5.1 (±3.1)
PL_1_c G1→a[#]	-	3.2 (±1.5)
PL_1_c W2→a[#]	-	3.8 (±2.2)
PL_1_c G3→a[#]	-	7.3 (±3.8)
PL_1_c S4→a[#]	-	6.8 (±3.2)
PL_1_c F5→a[#]	-	3.3 (±1.4)
PL_1_c F6→a[#]	-	2.0 (±1.3)
PL_1_c K7→a[#]	-	2.4 (±1.8)
PL_1_c K8→a[#]	-	4.3 (±3.1)
PL_1_c H11→a[#]	-	6.8 (±3.6)
PL_1_c V12→a[#]	-	6.0 (±4.0)
PL_1_c	-	1.8 (±0.5)
PL_1_c G1→a	-	4.1 (±0.9)
PL_1_c W2→a	-	2.0 (±1.3)
PL_1_c G3→a	-	1.4 (±0.6)
PL_1_c S4→a	-	1.7 (±0.3)
PL_1_c F5→a	-	1.8 (±0.5)
PL_1_c F6→a	-	2.6 (±0.6)
PL_1_c K7→a	-	6.2 (±2.5)
PL_1_c K8→a	-	5.0 (±2.3)
PL_1_c H11→a	-	3.4 (±0.7)
PL_1_c V12→a	-	1.6 (±0.4)

* $D_{E.\ coli}$ = Discriminatory binding capability for *E. coli* O157:H7 relative to *E. coli* ML35.

^ $D_{S.\ aureus}$ = Discriminatory binding capability for *E. coli* O157:H7 relative to *S. aureus*.

[#] Fragment not purified prior to screening.

Gray shading indicates confidence in discriminatory binding (D_{cell} is more than one standard deviation away from 1.0; the peptide binds to at least one organism).

Dash indicates not evaluated.

Appendix D: Screening Results of AMP-Inspired Sequences

Table D-1: Binding behavior of Markov chain sequences

Lot #	*E. coli* O157:H7	*S. aureus*	$D_{S.\ aureus}$*
NEP4-A01	0.030 (±0.031)	0.070 (±0.025)	0.4 (±0.5)
NEP4-A02	0.050 (±0.087)	0.074 (±0.038)	0.7 (±1.2)
NEP4-A03	0.048 (±0.045)	0.045 (±0.017)	1.1 (±1.1)
NEP4-A05	0.034 (±0.047)	0.052 (±0.019)	0.7 (±0.9)
NEP4-B02	0.000 (±0.064)	0.046 (±0.029)	0.3 (±0.6)
NEP4-B06	0.000 (±0.061)	0.000 (±0.045)	N/A
NEP4-B10	0.000 (±0.027)	0.111 (±0.099)	0.2 (±0.4)
NEP4-C01	0.111 (±0.099)	0.082 (±0.067)	1.4 (±1.6)
NEP4-C02	0.001 (±0.019)	0.000 (±0.025)	N/A
NEP4-C03	0.044 (±0.049)	0.085 (±0.039)	0.5 (±0.6)
NEP4-C05	0.037 (±0.030)	0.076 (±0.034)	0.5 (±0.5)
NEP4-C06	0.000 (±0.035)	0.020 (±0.024)	0.8 (±0.4)
NEP4-C08	0.007 (±0.137)	0.041 (±0.080)	0.2 (±3.3)
NEP4-C09	0.135 (±0.195)	0.213 (±0.080)	0.6 (±0.9)
NEP4-C12	0.128 (±0.062)	0.035 (±0.043)	3.7 (±4.9)
NEP4-D01	0.122 (±0.050)	0.082 (±0.056)	1.5 (±1.2)
NEP4-D02	0.000 (±0.026)	0.017 (±0.053)	0.1 (±0.04)
NEP4-D05	0.062 (±0.076)	0.037 (±0.028)	1.6 (±2.4)
NEP4-D06	0.013 (±0.110)	0.095 (±0.042)	0.1 (±1.2)
NEP4-D07	0.052 (±0.141)	0.071 (±0.043)	0.7 (±2.0)
NEP4-D10	0.096 (±0.138)	0.032 (±0.045)	3.0 (±6.1)
NEP4-D11	0.056 (±0.073)	0.021 (±0.021)	2.7 (±6.6)
NEP4-E01	0.000 (±0.057)	0.000 (±0.006)	N/A
NEP4-E02	0.118 (±0.029)	0.041 (±0.055)	2.9 (±4.0)
NEP4-E04	0.023 (±0.063)	0.026 (±0.042)	0.9 (±2.8)
NEP4-E05	0.177 (±0.028)	0.064 (±0.007)	2.8 (±0.5)
NEP4-E07	0.114 (±0.055)	0.040 (±0.033)	2.9 (±2.7)
NEP4-E08	0.000 (±0.035)	0.000 (±0.035)	N/A
NEP4-E11	0.000 (±0.082)	0.048 (±0.046)	0.1 (±0.5)
NEP4-F01	0.221 (±0.255)	0.054 (±0.056)	4.1 (±6.3)
NEP4-F02	0.000 (±0.124)	0.000 (±0.052)	N/A
NEP4-F03	0.328 (±0.292)	0.256 (±0.070)	1.3 (±1.2)
NEP4-F05	0.009 (±0.025)	0.008 (±0.018)	1.3 (±4.5)
NEP4-F06	0.178 (±0.248)	0.024 (±0.026)	7.4 (±13.0)
NEP4-F09	0.045 (±0.049)	0.018 (±0.016)	2.5 (±3.5)
NEP4-F11	0.000 (±0.006)	0.006 (±0.039)	N/A
NEP4-G02	0.190 (±0.209)	0.000 (±0.042)	N/A
NEP4-G03	0.035 (±0.072)	0.098 (±0.056)	0.4 (±0.8)
NEP4-G05	0.133 (±0.137)	0.071 (±0.019)	1.9 (±2.0)
NEP4-G07	-	0.000 (±0.044)	-
NEP4-G08	0.150 (±0.287)	0.050 (±0.001)	3.0 (±5.7)

Lot #	*E. coli* O157:H7	*S. aureus*	$D_{S.\ aureus}$*
NEP4-H01	0.142 (±0.224)	0.044 (±0.012)	3.2 (±5.2)
NEP4-H02	0.199 (±0.258)	0.149 (±0.081)	1.3 (±1.9)
NEP4-H04	0.010 (±0.113)	0.023 (±0.111)	0.4 (±5.3)
NEP4-H05	0.000 (±0.012)	0.222 (±0.185)	0.3 (±0.4)
NEP4-H06	0.000 (±0.133)	0.093 (±0.108)	0.0 (±1.4)
NEP4-H07	0.306 (±0.278)	0.076 (±0.100)	4.0 (±6.4)
NEP4-H10	0.205 (±0.252)	0.090 (±0.031)	2.3 (±2.9)
NEP5-F12	-	0.018 (±0.022)	-
NEP5-G01	-	0.057 (±0.038)	-
NEP5-G05	-	0.105 (±0.022)	-
NEP5-G07	-	0.000 (±0.012)	-
NEP5-G10	-	0.046 (±0.007)	-
NEP5-G11	-	0.000 (±0.003)	-

* $D_{S.\ aureus}$ = Discriminatory binding capability for *E. coli* O157:H7 relative to *S. aureus*. Values ≤ 1 indicate higher binding to *S. aureus.*

N/A = No detectable binding to one or both organisms.

Gray shading under the *E. coli* O157:H7 and *S. aureus* columns indicates confidence that peptide binds to target organism (confidence defined as mean binding value ≥ 0.050; mean binding value minus one standard deviation ≥ 0.020). Gray shading under the $D_{S.\ aureus}$ column indicates confidence in discriminatory binding (D_{cell} is more than one standard deviation away from 1.0; the peptide binds to at least one organism).

Dash indicates not evaluated.

Table D-2: Binding behavior of 2nd-generation Markov chain sequences

Library	Lot #	*S. aureus* binding
Alanine variants of NEP4-F03	NEP5-A04	0.021 (±0.034)
	NEP5-A05	0.111 (±0.074)
	NEP5-A06	0.025 (±0.020)
	NEP5-A12	0.011 (±0.026)
	NEP5-B01	0.000 (±0.020)
	NEP5-B03	0.060 (±0.055)
	NEP5-B04	0.000 (±0.034)
	NEP5-B05	0.012 (±0.038)
Other variants of NEP4-F03	NEP5-A01	0.000 (±0.037)
	NEP5-A02	0.000 (±0.038)
	NEP5-A03	0.038 (±0.049)
	NEP5-A07	0.049 (±0.018)
	NEP5-A08	0.057 (±0.021)
	NEP5-A11	0.000 (±0.013)
	NEP5-B06	0.018 (±0.011)
	NEP5-B07	0.010 (±0.027)
	NEP5-B08	0.048 (±0.059)
	NEP5-B09	0.021 (±0.039)
	NEP5-B10	0.064 (±0.032)
	NEP5-B11	0.080 (±0.047)
	NEP5-B12	0.000 (±0.001)
	NEP5-C02	0.000 (±0.022)
	NEP5-C03	0.000 (±0.010)
	NEP5-C04	0.038 (±0.016)
Charge/hydrophobicity-constrained sequences	NEP5-E02	0.101 (±0.036)
	NEP5-E03	0.066 (±0.050)
	NEP5-E04	0.071 (±0.026)
	NEP5-E07	0.037 (±0.019)
	NEP5-E08	0.028 (±0.019)
	NEP5-E09	0.059 (±0.008)
	NEP5-E10	0.217 (±0.077)
	NEP5-E12	0.116 (±0.013)
	NEP5-F02	0.061 (±0.013)
	NEP5-F03	0.049 (±0.013)
	NEP5-F04	0.058 (±0.020)
	NEP5-F05	0.034 (±0.016)
	NEP5-F06	0.109 (±0.030)
	NEP5-F07	0.008 (±0.004)
	NEP5-F09	0.017 (±0.002)
	NEP5-F10	0.011 (±0.019)
	NEP5-F11	0.072 (±0.009)
Sequences with double- and triple-lysine motifs	NEP5-G12	0.003 (±0.022)
	NEP5-H01	0.304 (±0.020)

Library	Lot #	*S. aureus* binding
Sequences with double- and triple-lysine motifs	NEP5-H02	0.000 (±0.015)
	NEP5-H03	0.059 (±0.017)
	NEP5-H04	0.101 (±0.017)
	NEP5-H06	0.083 (±0.009)
	NEP5-H07	0.047 (±0.021)
	NEP5-H08	0.030 (±0.009)
	NEP5-H09	0.102 (±0.008)
	NEP5-H11	0.270 (±0.043)
	NEP5-H12	0.059 (±0.017)

Gray shading indicates confidence that peptide binds to target organism (confidence defined as mean binding value ≥ 0.050; mean binding value minus one standard deviation ≥ 0.020).